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Alterations in HIV-1 Rev Transport in Response to Cell Stress

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Movement of HIV-1 Rev between the nucleus and cytoplasm is essential to its function. While normally nuclear, the protein can be induced to accumulate in the cytoplasm upon inhibition of RNA polymerase I/II. Nuclear accumulation of Rev in the presence of these inhibitors was found to be rescued upon addition of leptomycin B, an inhibitor of Rev nuclear export. This finding, in conjunction with kinetic data on nuclear import, indicates that the effect of the RNA polymerase inhibitors is due to an inversion of the rates of nuclear import versus export possibly achieved by increasing the rate of Rev nuclear export. We also examined whether changes in Rev localization could be due to a stress response. While neither ultraviolet radiation nor heat shock affected Rev subcellular localization, both oxidative and osmotic shocks induce changes in Rev localization comparable to that observed with the RNA polymerase inhibitors. The ability of certain serine/threonine kinase inhibitors, including CKI/II inhibitors, to cause cytoplasmic accumulation of Rev suggested that the alteration in Rev distribution could be due to changes in Rev or CRM1 phosphorylation. However, no change in extent of phosphorylation of either protein is observed upon treatment of cells with any of the agents tested, indicating involvement of another cellular factor.

INTRODUCTION

The regulation of RNA metabolism at the levels of splicing and RNA transport is critical to the replication of the human immunodeficiency virus (HIV-1). The 9-kb primary transcript of HIV-1 can be processed into unspliced, singly spliced, or doubly spliced transcripts to generate >25 mRNAs (Schwartz *et al.,* 1990; Wong-Staal, 1990). In addition to control at the level of RNA splicing, transport of viral RNA is also regulated (Emerman *et al.,* 1989; Felber *et al.,* 1989; Hammarskjöld *et al.,* 1989; Malim *et al.,* 1989b). The unspliced and singly spliced mRNAs are sequestered in the nucleus in the absence of an essential accessory protein of HIV, Rev (Sodroski *et al.,* 1986; Feinberg *et al.,* 1986). Rev transports the unspliced and singly spliced mRNAs to the cytoplasm, where they are translated into the structural proteins of the virus. Rev accomplishes this task through the action of various functional domains: (1) a nuclear localization signal (NLS) that directs Rev import into the nucleus (Cochrane *et al.,* 1990b; Malim *et al.,* 1989a; Perkins *et al.,* 1989; Hope *et al.,* 1990), (2) a RNA binding region that interacts with its target HIV RNA in the nucleus (Cochrane *et al.,* 1990a; Hadzopoulou-Cladaras *et al.,* 1989; Heaphy *et al.,* 1990; Malim *et al.,* 1989b; Rosen *et al.,* 1988; Zapp and Green, 1989), and (3) a nuclear export signal (NES) that targets the Rev-RNA complex to the cytoplasm (Fischer *et al.,* 1995; Meyer *et al.,* 1996). The presence of both an NLS and NES permits Rev to shuttle between the nucleus and cytoplasm of a cell, but it accumulates primarily in the nucleus and nucleolus (Cochrane *et al.,* 1990b; Malim *et al.,* 1989a; Perkins *et al.,* 1989; Hope *et al.,* 1990). However, various inhibitors of RNA polymerase I and II, such as actinomycin D (Act. D), dichlorobenzimidizole riboside (DRB), or mycophenolic acid (MPA), cause Rev to redistribute to the cytoplasm, leaving little Rev staining in the nucleus (D'Agostino *et al.,* 1995; Kalland *et al.,* 1994; Meyer and Malim, 1994; Richard *et al.,* 1994; Wolff *et al.,* 1995). Redistribution to the cytoplasm under these conditions requires a functional NES (Meyer and Malim, 1994; Stauber *et al.,* 1995; Szilvay *et al.,* 1995).

Subcellular localization is one of many means of regulating protein activity (Turpin *et al.,* 1999; Vandromme *et al.,* 1996). Several proteins dramatically alter their subcellular localization in response to cell stresses such as heat or osmotic shock (Hood and Silver, 1999). Changes in localization can have significant effects on a protein's activity, altering its ability to interact with substrates or inhibitors. Given the requirement for Rev to continuously shuttle between the nucleus and cytoplasm, it is of interest to understand how Rev subcellular localization is regulated. Nucleocytoplasmic shuttling of Rev involves interaction with soluble cellular factors that mediate Rev's interaction with the nuclear pore complex (NPC) and execute transport through the nuclear pore. In the cytoplasm, the arginine-rich NLS interacts with an import receptor, importin β, which docks onto the NPC (Truant and Cullen, 1999; Palmer and Malim, 1999). In the nu-

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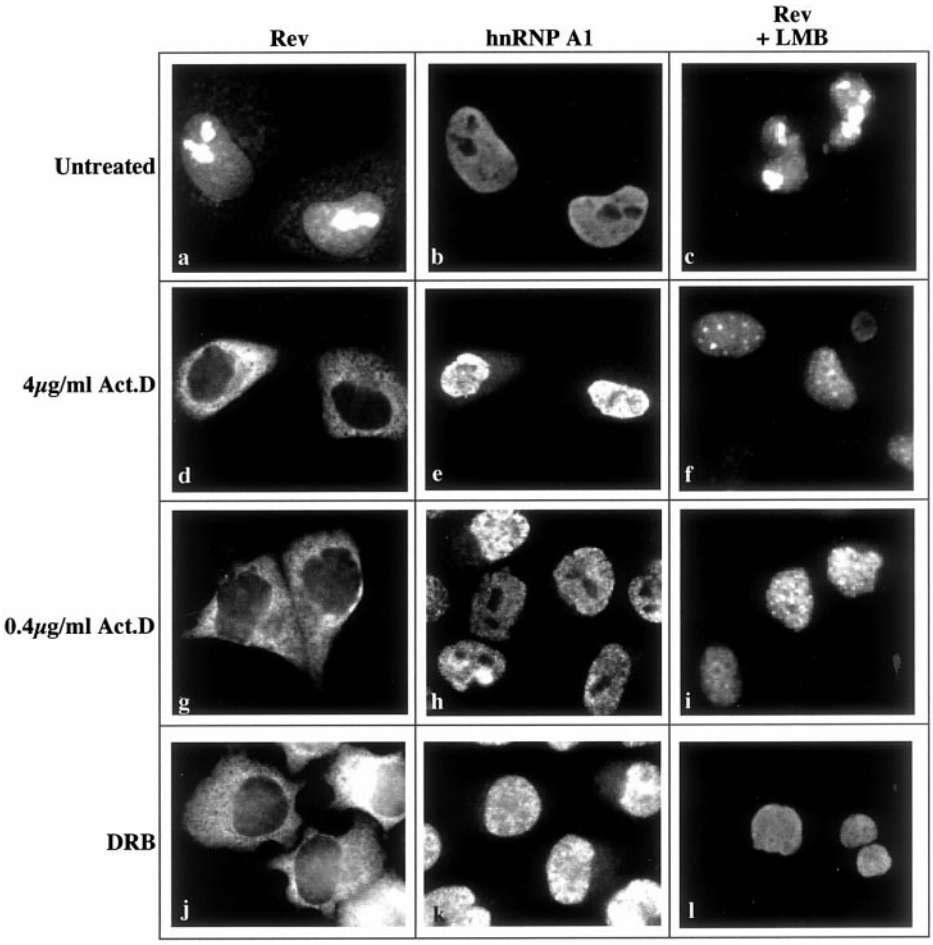

FIG. 1. Rev accumulation in the cytoplasm is due to a change in relative rates of nuclear import versus export. Rev redistributes to the cytoplasm in response to RNA polymerase I/II inhibitors (left) but re-accumulates in the nucleus upon subsequent addition of LMB (right). HeLaRev cells were untreated (a, b, and c), or treated for 1 h with 4 μg/ml Act. D (d, e, and f), 0.4 μg/ml Act. D (g, h, and i), or 150 μM DRB (j, k, and l). Rev re-accumulated in the nucleus of Act. D (f and i)- or DRB (l)-treated cells upon addition of 200 nM LMB to the media for 1 h. Cells were co-stained for hnRNPA1 (middle) and treated as indicated. Upon RNA polymerase II inhibition by 4 μg/ml Act. D, a slight increase in cytoplasmic hnRNPA1 is visible.

cleus, the leucine-rich NES of Rev interacts with an export receptor, CRM1 (exportin-1), which docks onto the NPC in a Ran-GTP-dependent manner (Fornerod *et al.,* 1997; Fischer *et al.,* 1995). The antifungal antibiotic leptomycin B (LMB) blocks the interaction between the Rev NES and CRM1, preventing nuclear export of Rev (Wolff *et al.,* 1997).

To further understand the mechanism underlying Rev's redistribution in response to inhibition of RNA polymerase I/II, we used LMB to demonstrate that redistribution induced by the polymerase inhibitors is reversible, the result of an alteration in the relative rates of import and export. We also established that oxidative and osmotic shocks as well as CKI/II inhibitors induce changes in Rev localization comparable to that observed with the RNA polymerase inhibitors. Effects of the kinase inhibitors on Rev subcellular localization can be ascribed to an alteration in the relative rates of transport into and out of the nucleus given the reversibility of their effect upon treatment with LMB. Neither Rev nor CRM1 show alteration in phosphorylation upon treatment of cells associated with changes in Rev subcellular distribution, suggesting that the effect of the kinase inhibitors is mediated by an as yet unidentified cellular target, which regulates nucleocytoplasmic trafficking.

RESULTS

Cytoplasmic accumulation of Rev is reversed upon leptomycin B addition

Rev accumulates in the cytoplasm of cells treated with the RNA polymerase inhibitors Act. D and DRB (D'Agostino *et al.,* 1995; Kalland *et al.,* 1994; Meyer and Malim, 1994; Richard *et al.,* 1994; Wolff *et al.,* 1995) (also, Fig. 1). To ascertain whether Rev accumulation in the cytoplasm of treated cells is due to changes in the activity of its NLS, an inhibitor of Rev nuclear export, leptomycin B (LMB), was tested for its effect on Rev localization. HeLaRev cells were treated with Act. D for 1 h, at which point LMB was added to the media for 1 h.

TABLE 1

Kinetics of Leptomycin B Reversal of Actinomycin D-Induced Accumulation of Rev in the Cytoplasm

	Rev subcellular distribution		
Time after Lept. B addition (min)	N > C (%)	N = C (%)	N < C (%)
0	0	49	51
10	66	31.8	2.2
15	74	26	0
20	80	20	0
30	68	32	0

Note. HeLa Rev cells were treated for 2 h with 4 μg Act. D/ml and 20 μg/ml cycloheximide at which time leptomycin B was added to a final concentration of 200 nM. Cells were fixed at the indicated times after leptomycin B addition and the subcellular localization of Rev determined by immunofluorescence. Data shown is derived from scoring a minimum of 100 cells per time point as to the pattern of Rev distribution observed and is representative of 4 independent trials. N > C significant accumulation of Rev in the nucleus, little or no staining apparent in the cytoplasm. N = C equivalent staining of Rev in the nucleus and cytoplasm. N < C accumulation of Rev in the cytoplasm with little or no staining in the nucleus.

As shown in Fig. 1, Rev within the cytoplasm of Act. D-treated cells is capable of nuclear import as subsequent addition of LMB results in accumulation of Rev in the nucleus. Nuclear re-accumulation of Rev upon addition of LMB was complete in 10 min (Table 1). The rapid nuclear import of Rev in the presence of Act. D and LMB suggests that cytoplasmic accumulation of the protein is due to an inversion in the relative rates of Rev nuclear export versus nuclear import. Additional tests demonstrated that the kinetics of Rev nuclear accumulation upon addition of LMB was the same in the presence of its target RNA (data not shown), indicating that formation of the RNA–protein complex does not affect Rev shuttling behaviour.

In support of the conclusion that cytoplasmic accumulation of Rev is not attributable to activation of cytoplasmic retention or inactivation of its nuclear import, the SV40 large T antigen NLS (SVNLS) was fused to the amino terminus of Rev, creating SVNLSRev (Fig. 2A). Recent studies (Truant and Cullen, 1999; Palmer and Malim, 1999) have determined that both the NLS of Rev and the SV40 large T antigen share a common factor in their mechanism of import, importin β. Consequently, if changes in Rev distribution are attributable to changes in rates of import, the SV40 NLS may be equally affected. In contrast to Rev, SVNLSRev accumulates in the nucleus in the presence of Act. D or DRB (Fig. 2B). If cytoplasmic accumulation of Rev had been due to anchoring of the protein in the cytoplasm rather than alteration in transport rates, the SVNLS would not be able to rescue Rev import into the nucleus. Subsequent heterokaryon assays show that SVNLSRev equilibrates between human and mouse nuclei of fused cells (Fig. 2C) confirming the ability of the fusion protein to shuttle. Finally, SVNLSRev was found to induce Rev-dependent expression to a similar extent as unmodified Rev (data not shown). Thus, addition of the SVNLS does not appear to affect Rev tertiary folding or its ability to shuttle but does rescue Rev nuclear accumulation in Act. D-treated cells.

To determine whether the inversion of Rev transport rates is due to an increase in Rev export rate alone or an attendant decrease in Rev import, a washout experiment was performed. The effects of DRB are reversible upon washing of the cells (Meyer and Malim, 1994; Richard *et al.,* 1994). HeLaRev cells treated with DRB to re-localize Rev to the cytoplasm were (1) subsequently treated with LMB in the continued presence of DRB or (2) washed with PBS to remove DRB from the cells, and media containing LMB then was added. The former group of cells measures nuclear import under conditions where nuclear export dominates due to the presence of DRB, while the latter measures nuclear import in the absence of DRB and, thus, akin to when nuclear import dominates. Two doses of DRB were tested to determine whether residual drug present following washing could be affecting the import rates measured. As presented in Fig. 3, nuclear re-accumulation of Rev is completed in 3 min under all conditions tested, with no discernible difference in the rate of Rev nuclear accumulation in the absence or presence of RNA polymerase inhibitors.

Redistribution of Rev is also induced by oxidative and osmotic shock

The accumulation of Rev in the cytoplasm of cells treated with Act. D, DRB, or MPA has been previously attributed to their common effect, inhibition of RNA polymerase I (D'Agostino *et al.,* 1995; Kalland *et al.,* 1994; Meyer and Malim, 1994; Richard *et al.,* 1994; Wolff *et al.,* 1995). However, the subcellular distribution of several proteins is altered in response to various stresses on the cell (Gorner *et al.,* 1998; Ducret *et al.,* 1999; Hood and Silver, 1999). To ascertain whether the effect of these RNA polymerase inhibitors may be mediated by induction of a stress response, the effect of UV stress, heat shock, oxidative stress, or hyperosmotic shock on Rev subcellular distribution was examined. As shown in Fig. 4, treatment of Rev-expressing cells for up to 1 h with UV or 3 h of heat shock (42°C) had no effect on Rev subcellular distribution. In contrast, oxidative stress induced by treatment with 4 mM hydrogen peroxide or osmotic stress induced by 0.6 M sorbitol treatment did result in the cytoplasmic accumulation of Rev. Relocalization was complete within 30 min of treatment comparable to that seen in the presence of Act. D. The non-shuttling nuclear protein hnRNP C remained nuclear under all conditions (data not shown), demonstrating that nuclear integrity persists over the course of these treatments.

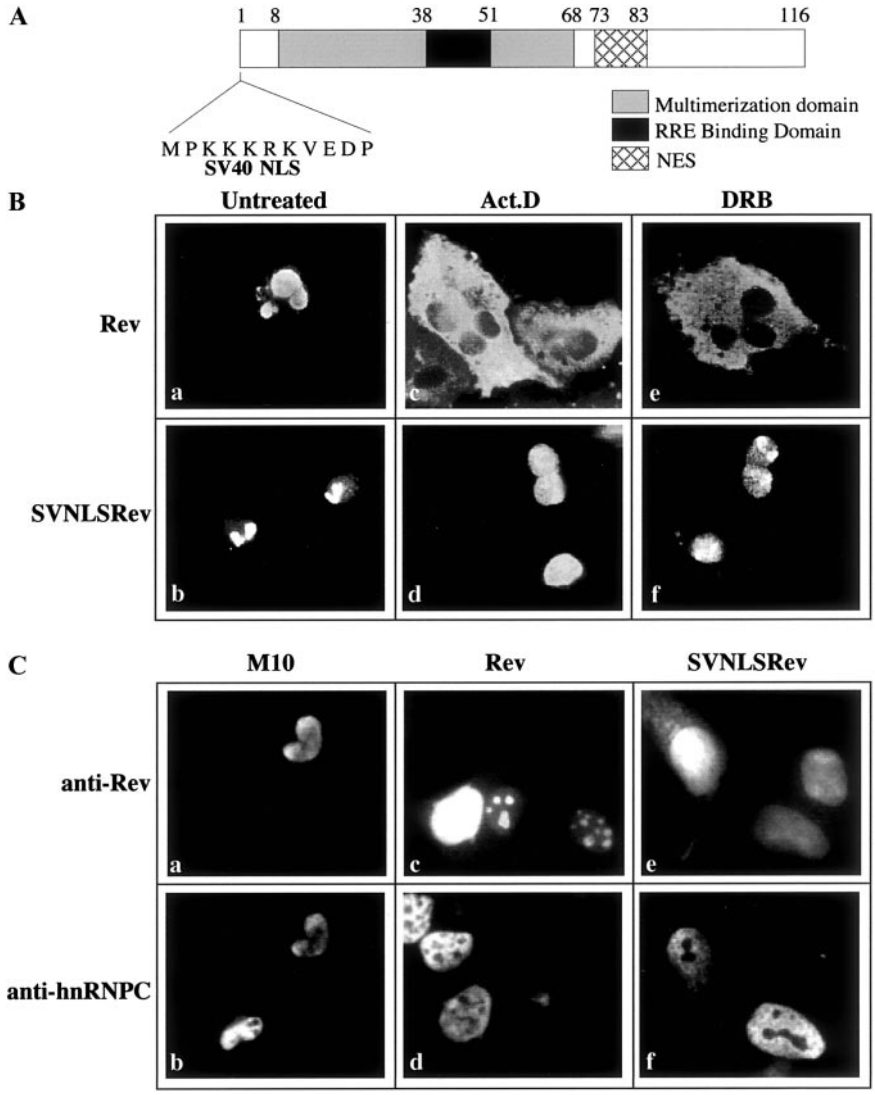

FIG. 2. Addition of the SV40 large T antigen NLS to Rev rescues its nuclear import in the presence of RNA polymerase inhibitors. (A) Schematic of SVNLSRev. (B) SVNLSRev remains nuclear upon Act. D or DRB treatment. Cos-7 cells were transfected with Rev (a, c, and e) or SVNLSRev (b, d, and f) and their subcellular distribution determined by indirect immunofluorescence 48 h later (a and b), or after 2 h of treatment with 4 μg/ml Act. D (c and d) or 150 μM DRB (e and f). Treatments were done in the presence of cycloheximide. (C) SVNLSRev is capable of nucleocytoplasmic shuttling as it enters the nuclei of mouse cells fused to human cells transfected with SVNLSRev. HeLa cells were transfected with RevM10 (a and b), Rev (c and d), or SVNLSRev (e and f) and then fused with mouse 3T3 cells by PEG treatment. The cells were stained with antibody to Rev (a, c, and e) and human hnRNP C (b, d, and f). Both Rev and SVNLSRev shuttle between the nucleus and cytoplasm of the cell. They can be seen to accumulate in nuclei of mouse cells fused to human cells (indicated by staining for hnRNP C) expressing the protein. M10, harboring a mutation in its NES rendering it unable to exit to nucleus, does not shuttle and cannot be found in mouse nuclei.

HnRNP A1, another shuttling protein whose transport pathways are distinct from Rev also has an altered distribution pattern in response to Act. D treatment (Pinol-Roma and Dreyfuss, 1992). However, hnRNP A1 redistribution is specific to inhibition of RNA polymerase II: α-amanitin and only high doses (4 μg/ml) of Act. D induce a slight increase in its cytoplasmic signal (Fig. 1E). To ascertain whether hnRNP A1 also responds to oxidative or osmotic stress, cells were treated with hydrogen peroxide or sorbitol and co-stained for Rev and hnRNP A1. In agreement with recent findings (van der Houven van Oordt *et al.,* 2000), hnRNP A1 subcellular localization is altered upon osmotic shock but not upon any of the other stresses (Fig. 4). While some cytoplasmic accumulation of hnRNP A1 is discernable at 0.6 M sorbitol, higher sorbitol concentrations were found to elicit a more dramatic shift in the subcellular localization of the protein (Fig. 4).

Redistribution of Rev induced by kinase inhibitors

Signaling cascades induced in response to various stresses involve alteration of the activity of various phosphatases and kinases, in particular, one of two stress-

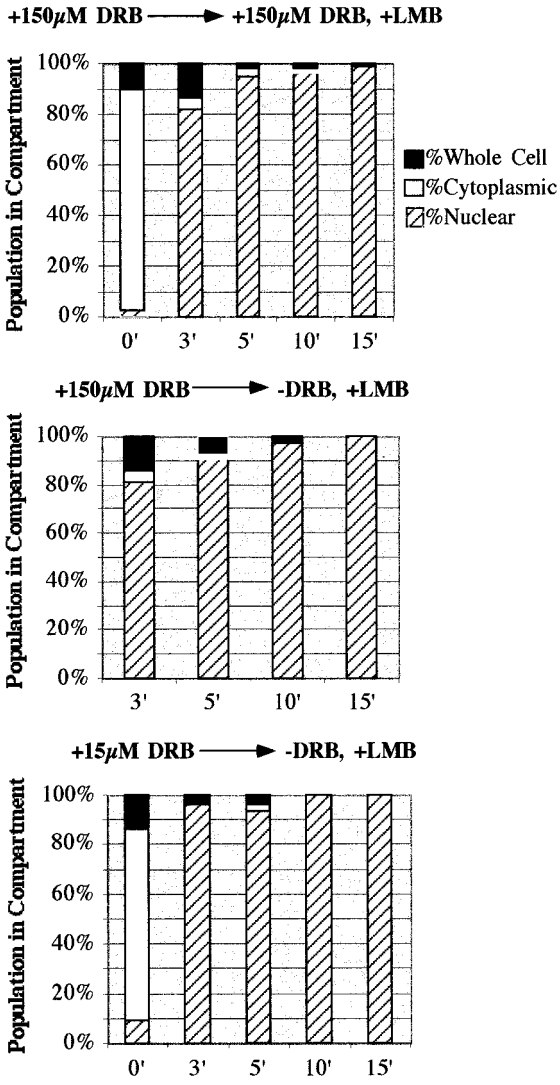

FIG. 3. The rate of Rev nuclear import is not altered upon DRB treatment. For the unwashed set, HeLaRev cells were treated with 150 μM DRB for 2 h and then 200 nM LMB added for 3–15 min, as indicated. The unwashed set is representative of nuclear import in the presence of DRB. For the washed set, HeLaRev cells were treated with 150 or 15 μM DRB to accumulate Rev in the cytoplasm, washed 4× with media, and then 200 nM LMB added for 3–15 min, as indicated. The washed cells are representative of nuclear import of Rev in the absence of inhibitor. For each condition, fields of 100 cells were scored as indicated in Table 1 (primarily cytoplasmic, nuclear, or whole cell). The bar graphs show that nuclear re-accumulation of Rev upon LMB addition occurs within 3 min for both.

activated protein kinase pathways (Toone and Jones, 1998; Ichijo, 1999; Tibbles and Woodgett, 1999). In an attempt to identify kinases or phosphatases modulated by hydrogen peroxide or sorbitol involved in signaling Rev redistribution, HeLaRev cells were treated with a variety of phosphatase inhibitors (okadaic acid and sodium orthovanadate), kinase inhibitors (herbimycin A, PD98059, wortmannin, genistein, staurosporine, A3, SB 203580, and H89), and an activator of the stress-induced kinase p38 (anisomycin) (Cano *et al.,* 1996). Table 2 outlines the drugs, the category to which they belong, and the concentrations tested for up to 2 h. All drugs were tested (1) alone for their effect on Rev subcellular localization, (2) prior to Act. D, hydrogen peroxide or sorbitol treatment to determine whether relocalization could be inhibited, or (3) after Act. D, hydrogen peroxide and sorbitol treatment to observe whether relocalization could be reversed. None of the drugs were found to block or reverse the effects of Act. D, hydrogen peroxide, or sorbitol treatment on Rev subcellular distribution.

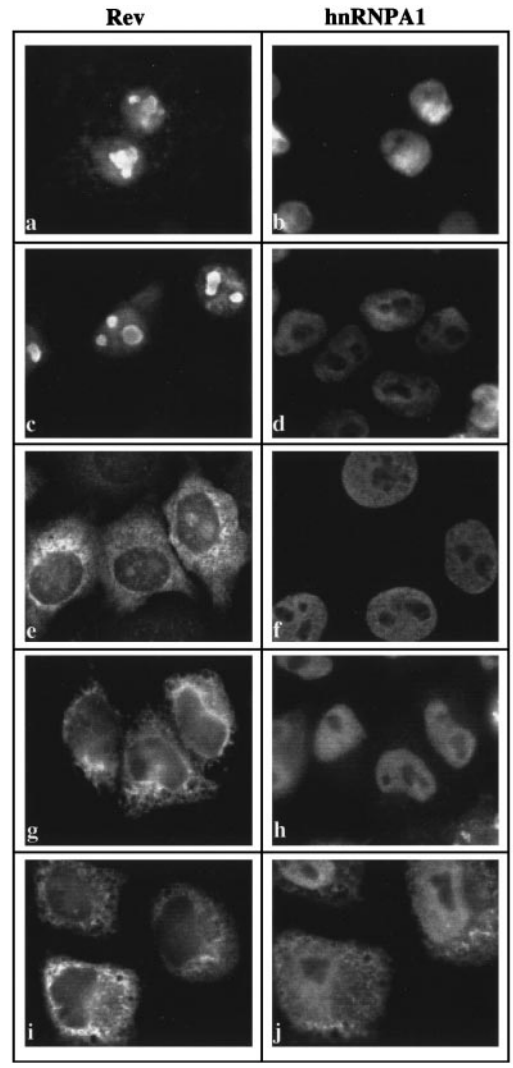

FIG. 4. Oxidative and osmotic shock induce a redistribution in Rev. Subcellular localization of Rev (left) and hnRNP A1 (right) in HeLaRev cells in response to various shocks. Neither 1 h UV shock (200 J/m2; a and b) nor 3 h heat shock (42°C; c and d) have any effect on Rev or hnRNP A1 distribution. One-hour treatment with 4 mM hydrogen peroxide (e and f) induces cytoplasmic accumulation of Rev but not hnRNP A1. Osmotic shock by treatment with 0.6 M sorbitol (g and h) causes cytoplasmic accumulation of Rev and increases cytoplasmic signal of hnRNP A1, while 0.8 M sorbitol dramatically increases the amount of hnRNP A1 in the cytoplasm (i and j). None of the treatments affected hnRNP C subcellular distribution (data not shown). All treatments were performed in the presence of 20 μg/ml cycloheximide.

TABLE 2

Drugs Tested for their Effect on Rev Subcellular Distribution

Class	Drug	Properties (IC50)	Doses tried	Rev localization
MAP kinase activator	Anisomycin	Activates p54 (JNK) and MAP kinases; involved in activation of SAPKs	1 mM	Nuclear, nucleolar
Phosphatase inhibitor	Okadaic acid	PP2A = 0.1 nM PP1 = 10–15 nM PP2B = 5 μM	10 μM	Nuclear, nucleolar
	Sodium orthovanadate	Potent inhibitor of alkaline phosphatase; inhibitor of tyrosine phosphatases of general/broad specificity	100 μM	Nuclear, nucleolar
Kinase inhibitors	Herbimycin A	p60v-src = 900 nm no effect on PKC or PKA	10 μM	Nuclear, nucleolar
	PD98059	MEK1 = 5–10 μM MEK2 = 50 μM	500 μM	Nuclear, nucleolar
	SB203580	p38 kinase = 600 nM	100 μM	Nuclear, nucleolar
	Wortmannin	MLCK = 0.2 μM	2 μM	Nuclear, nucleolar
	Genistein	EGFR kinase = 2.6 μM	10 μM	Nuclear, nucleolar
		p60v-src = 25 μM	**50 μM**	**Cytoplasmic**
		PKC, PKA, S/T kinases > 100 μM	**100 μM**	**Cytoplasmic**
	Staurosporine	MLCK = 0.0013 μM		
		PKA = 0.007 μM	10 μM	Nuclear, nucleolar
		PKC = 0.0007 μM	**100 μM**	**Cytoplasmic**
		PKG = 0.0085 μM		
		CaM kinase = 0.02 μM		
	DRB 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole	CKII = 6 μM	**150 μM**	**Cytoplasmic**
			15 μM	**Cytoplasmic**
			6 μM	**Whole cell to cytoplasmic**
	H89	PKA = 0.048 μM		
		PKG = 0.48 μM		
		MLCK = 28.3 μM	50 μM	Nuclear, nucleolar
		PKC = 31.7 μM		
		CKI = 38.3 μM	**140 μM**	**Cytoplasmic**
		CKII = 137 μM		
	A3	PKG = 3.8 μM		
		PKA = 4.3 μM	10 μM	Nuclear, nucleolar
		CKII = 5.1 μM	**80 μM**	**Cytoplasmic**
		MLCK = 7.4 μM		
		PKC = 47 μM		
		CKI = 80 μM		

Note. Drugs and dose ranges tested for their effect on Rev subcellular distribution. All drugs were tested for their effect on Rev in HeLaRev cells alone, prior to and after Act. D or stress treatment. Indicated in bold are the doses of the corresponding drug that caused cytoplasmic accumulation of Rev. IC50 values cited are derived from the Calbiochem Signal Transduction Catalog and Technical Resource. 1998. Calbiochem-Novabiochem Corp.

However, two kinase inhibitors, genistein and staurosporine, did elicit an accumulation of Rev in the cytoplasm but only at concentrations at which they are general, non-specific inhibitors of serine/threonine kinases (Table 2, Fig. 5A). Like oxidative stress, neither genistein nor staurosporine induced any changes in hnRNP A1 or C subcellular localization (data not shown).

Previously, DRB-induced alteration of Rev subcellular distribution had used doses between 100 and 150 μM (D'Agostino *et al.,* 1995; Kalland *et al.,* 1994; Meyer and Malim, 1994; Richard *et al.,* 1994; Wolff *et al.,* 1995). However, DRB has been shown to be a potent and specific inhibitor of the serine/threonine kinase, CKII, at much lower concentrations (Zandomeni *et al.,* 1986). Therefore, DRB was tested for its ability to induce cytoplasmic accumulation of Rev at 15 and 6 μM in a 2-h time span. These concentrations were found to be sufficient to cause the redistribution of Rev from the nucleus to the cytoplasm in the majority of cells (Fig. 5A). In light of these results, other inhibitors of CKII were then tested for their effect on Rev distribution. Both A3 and H89, inhibitors of CKI and CKII (Hidaka and Kobayashi, 1992; Inagaki *et al.,* 1986), induced the cytoplasmic accumulation of Rev within 30 min (Fig. 5A). Subsequent tests in HeLa and Cos-7 cells transfected with SVH6Rev and SVH6M10 (Malim *et al.,* 1989a) confirmed that redistribution is not an attribute unique to the stable cell line, and is dependent on a functional NES (data not shown).

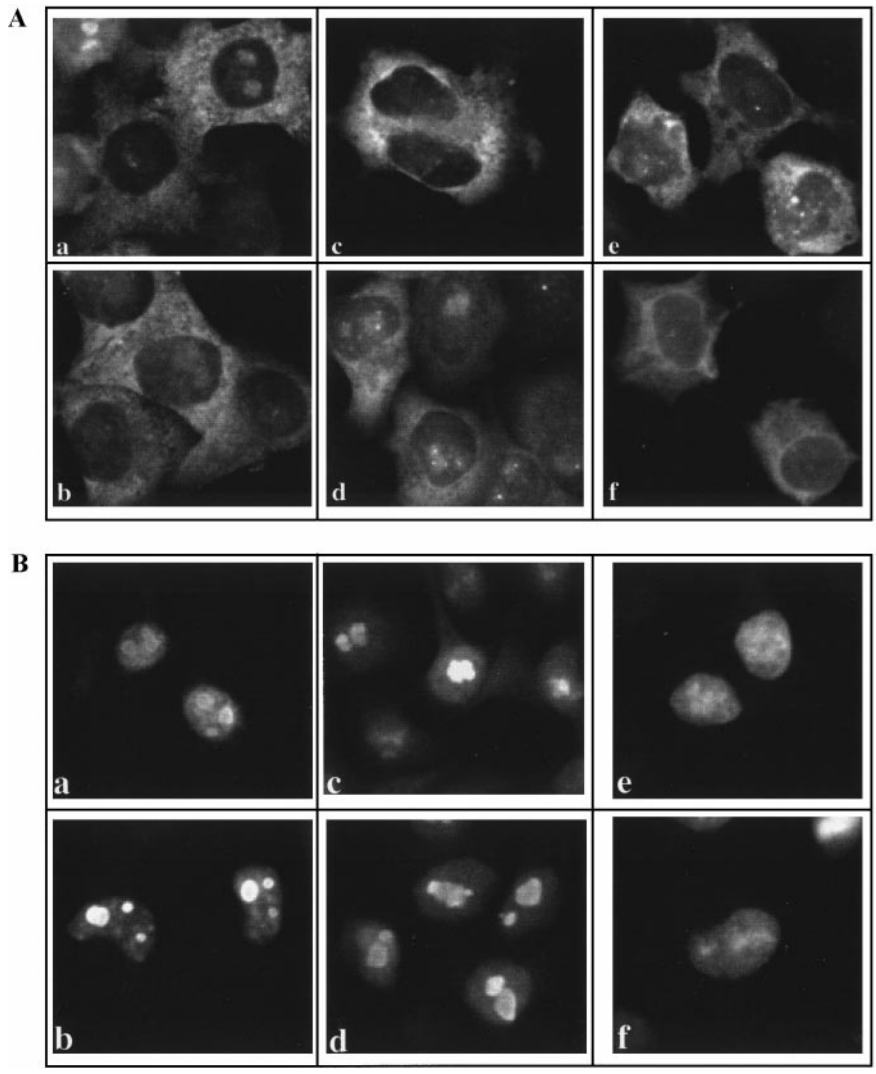

FIG. 5. Effect of kinase inhibitors on Rev localization. (A) HeLaRev cells were treated with 100 μM genistein (a), 100 μM staurosporine (b), 15 μM DRB (c), 6 μM DRB (d), 80 μM A3 (e), or 140 μM H89 (f) in the presence of cycloheximide for 1 h. Cells were then processed for immunofluorescence and stained for Rev. (B) Leptomycin B induces nuclear accumulation of Rev redistributed to the cytoplasm in response to the various stresses and kinase inhibitors. HeLaRev cells were treated for 1 h with 4 mM H_2O_2 (a), 0.6 M sorbitol (b), 100 μM genistein (c), 100 μM staurosporine (d), 80 μM A3 (e), or 140 μM H89 (f). LMB was then added to a final concentration of 200 nM for 15, 30, 45, or 60 min. Cells were then processed for immunofluorescence. Shown here are samples after 15-min treatment with LMB. All treatments were performed in the presence of cycloheximide.

The ability of LMB to reverse the effects of the various stresses and kinase inhibitors on Rev localization was also tested. As shown in Fig. 5B, cytoplasmic accumulation of Rev induced by the various drug treatments was reversed within 15 min of LMB addition. This is the same time span required for LMB reversal of Act. D effects on Rev localization.

Changes in Rev subcellular distribution does not correlate with changes in its phosphorylation or that of CRM1

The observation that serine/threonine kinase inhibitors can alter Rev localization, coupled with previous identification of Rev phosphorylation on serine residues (Cochrane *et al.,* 1989; Hauber *et al.,* 1988), suggested that the redistribution observed could be due to alterations in Rev modification. To determine whether there was a correlation between changes in Rev localization and phosphorylation, Rev-expressing cells were labeled with [^{32}P]orthophosphate and then treated with the inducers of Rev relocalization. Rev was immunoprecipitated from all the samples and compared for phosphorylation status. As can be seen in Fig. 6A, there is no alteration in the extent of Rev phosphorylation upon Act. D, DRB, A3, hydrogen peroxide, or sorbitol treatment that correlates with the changes in Rev subcellular distribution. CRM1 was also immunoprecipitated from these samples and did not show any changes in its phosphorylation status (Fig. 6B) under the conditions tested.

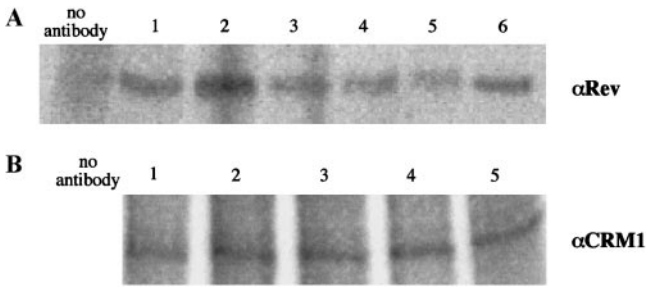

FIG. 6. Effect of treatments on Rev and CRM1 phosphorylation. (A) Rev immunoprecipitated from ^{32}P-labeled HeLaRev cells treated with various drugs. Shown is protein precipitated from cells that were untreated (lane 1) or treated for 1 h with 4 μg/ml Act. D (lane 2), 150 μM DRB (lane 3), 80 μM A3 (lane 4), 4 mM hydrogen peroxide (lane 5), or 0.6 M sorbitol (lane 6). (B) CRM1 immunoprecipitated from ^{32}P-labeled HeLaRev cells that were untreated (lane 1) or treated for 1 h with 4 μg/ml Act. D (lane 2), 150 μM DRB (lane 3), 80 μM A3 (lane 4), or 0.6 M sorbitol (lane 5).

DISCUSSION

A protein's activity and function can be regulated by alterations in its subcellular localization. Regulation of subcellular localization of a protein can be mediated by post-translational modifications, masking of its NLS or NES, or changes in its interacting partner(s) (Turpin *et al.,* 1999; Vandromme *et al.,* 1996). Many proteins that play a role in stress response are activated or inactivated by changes in their subcellular localization (Hood and Silver, 1999; Ducret *et al.,* 1999; Gorner *et al.,* 1998). Other stimuli induce subcellular redistribution of proteins include changes in intracellular calcium levels (Zhu and McKeon, 1999), cell cycle stage (Jin *et al.,* 1998), growth factors (Biggs *et al.,* 1999), phosphate or glucose starvation (Hood and Silver, 1999), and differentiation. Several proteins [Rev (D'Agostino *et al.,* 1995; Kalland *et al.,* 1994; Meyer and Malim, 1994; Richard *et al.,* 1994; Wolff *et al.,* 1995), hnRNP A1 (Pinol-Roma and Dreyfuss, 1992), HuR (Fan and Steitz, 1998), and VHL (Lee *et al.,* 1999)] that interact with RNA show transcription-dependent subcellular localization. Except for the latter example, inhibition of RNA polymerase I/II increases cytoplasmic levels of the normally nuclear factor.

Rev normally accumulates in the nucleus and nucleolus of cells. It was initially shown to be a shuttling protein through treatment of Rev-expressing cells with RNA polymerase I inhibitors such as Act. D (D'Agostino *et al.,* 1995; Kalland *et al.,* 1994; Meyer and Malim, 1994; Richard *et al.,* 1994; Wolff *et al.,* 1995). After such treatment, Rev is mainly found in the cytoplasm with little or no staining of the nucleus. However, the mechanism for Act. D- or DRB-induced redistribution of Rev was unknown. It could be due to a block in Rev nuclear import or an alteration in its rate of nuclear transport in favour of nuclear export. In this paper, we demonstrate that addition of LMB, an inhibitor of Rev nuclear export, to Act. D- or DRB-treated cells causes Rev to re-accumulate in the nucleus (Fig. 1). This result and the finding that addition of the SV40 NLS to Rev prevents its cytoplasmic accumulation in response to Act. D suggest that the accumulation of Rev in the cytoplasm in response to the inhibitors can be attributed to an inversion in the rates of nuclear import and nuclear export as Rev is still capable of nuclear accumulation. Under normal conditions, the continuously shuttling Rev accumulates in the nucleus, presumably due to a greater rate of Rev nuclear import than nuclear export. Upon Act. D or DRB treatment, Rev accumulates in the cytoplasm. Addition of the SV40 NLS blocks relocalization to the cytoplasm by virtue of the presence of a second, putatively stronger NLS, maintaining a high rate of import. By comparing the time span required for nuclear re-accumulation after LMB addition between DRB-treated cells and "washed" cells, we show that there is no discernible difference in the rate of nuclear import in the absence or presence of the drug (Fig. 3). That the rates observed following washing are not due to the presence of residual drug is suggested by the similarity in results using minimum doses of DRB (150 *vs* 15 μM DRB). These results suggest that the inversion in nuclear transport rates of Rev may be due to an increase in the rate of Rev nuclear export alone. However, it is also possible that it is attributable to an increase in nuclear export rate accompanied by a simultaneous decrease in the rate of nuclear import. More direct and precise measurement of the rate of Rev nuclear import will be required to discriminate between these two hypotheses.

In support of the former hypothesis is a study examining the control of the subcellular distribution of the von Hippel-Lindau tumor supressor gene (VHL). VHL normally resides in the cytoplasm and accumulates in the nucleus only upon RNA polymerase II inhibition (Lee *et al.,* 1999). An interesting aspect of this shuttling protein is that its nuclear accumulation upon inhibition of RNA polymerase II has been attributed to a decrease in its nuclear export rate. This pattern of regulation can be disrupted by the addition of the Rev NES to VHL. The fusion protein was found to accumulate in the cytoplasm both in the absence and presence of RNA polymerase inhibitors. Nuclear accumulation of the fusion protein in the presence of Act. D could be induced by addition of LMB. This observation suggests that the cytoplasmic accumulation of the VHL-NES protein was due to the high activity of the Rev NES under conditions when the nuclear import rate of VHL is normally dominant.

We also demonstrate in this paper that certain stresses can mimic the Act. D-induced cytoplasmic accumulation of Rev. Neither heat shock nor UV stress have an effect on Rev subcellular distribution (Fig. 4). In contrast, oxidative shock induced by hydrogen peroxide treatment and osmotic shock do induce Rev to accumulate in the cytoplasm. The effects observed are not attributable to a general release of nuclear factors as the

non-shuttling nuclear protein, hnRNP C, remains nuclear under these conditions. Similarly, the response observed cannot be attributed to effects on nucleolar function given that cells stressed or treated with the various drugs retained nucleolar organization and function (data not shown). That hnRNP A1 also shows increased cytoplasmic signal upon Act. D treatment (Pinol-Roma and Dreyfuss, 1992) or osmotic stress indicates that these treatments are having effects on multiple transport pathways. However, differences in the extent of protein relocalization and the conditions required to elicit the response suggest that the mechanisms regulating Rev and hnRNP A1 transport are distinct.

Exposure of cells to stressful conditions has been shown to elicit activation of a number of signaling pathways, the most predominant involving stress-activated protein kinases such as the JNK (c-Jun N-terminal kinase) and p38 MAP kinases (Toone and Jones, 1998; Ichijo, 1999; Tibbles and Woodgett, 1999). Consequently, various kinase activators/inhibitors and phosphatase inhibitors were examined for their effect on Rev subcellular distribution to probe the involvement of these pathways in the response of Rev to cell stress. None of the drugs tested inhibited or reversed the effects of Act. D, DRB, hydrogen peroxide, or sorbitol. Treatment of cells with the p38 activator anisomycin, the p38 inhibitor SB 203580, the inhibitor of MEK1, PD98059, or phosphatase inhibitors had no effect on Rev alone or Rev redistribution stimulated by Act. D or sorbitol. Of the various kinase inhibitors initially tested, only genistein and staurosporine had any effect on Rev. However, rather than inhibiting cytoplasmic accumulation of Rev, they induced it at concentrations far above their specificity for tyrosine kinases and at a level where a non-specific inhibition of kinase activity is observed. Given that DRB is a CKII inhibitor with an IC_{50} of 6 μM, lower dose ranges of DRB were tested to determine whether its effect on CKII is responsible for redistribution of Rev rather than its effect on RNA polymerases. As shown in Fig. 5A, both DRB and two other CKI/II inhibitors, A3 and H89, cause Rev redistribution. Similar to Act. D and DRB, reversal of the effects of hydrogen peroxide, sorbitol, and the kinase inhibitors that affect Rev redistribution was observed upon addition of LMB to the media. In all cases, LMB reversal was completed within 15 min (Fig. 5B). The similarity of the responses of Rev to the various treatments suggests that they share a mechanism for altering the relative rates of Rev import versus export. The specificity of these inhibitors for serine/threonine kinases and the previous identification of Rev as being modified on serine residues (Hauber *et al.,* 1988; Cochrane *et al.,* 1989) suggested the possibility that changes in Rev subcellular accumulation may correlate with changes in its phosphorylation. However, as demonstrated in Fig. 6, neither Rev nor CRM1 showed any changes in the extent of labeling upon treatment with Act. D, DRB, A3, hydrogen peroxide, or sorbitol which correlates with changes in Rev subcellular distribution. In further support of the conclusion that alteration of Rev phosphorylation is not involved in its redistribution, a deletion mutant of Rev lacking the primary phosphorylation sites (Rev84) (Cochrane *et al.,* 1989) was found to display similar responses and did not undergo changes in its phosphorylation (data not shown). Changes in Rev transport rates would therefore appear to be due to modification of some other cellular factor(s).

The response of Rev differs markedly from that recently reported for the Net repressor (Ducret *et al.,* 1999). Like Rev, the Net repressor is a shuttling protein containing an NES that interacts with CRM1. However, the predominately nuclear accumulation of Net is altered upon treatment of cells with anisomycin, U.V. and heat shock but not with hyperosmotic stress (0.6 M sorbitol). The response of Net to stress was subsequently shown to be dependent upon JNKK (c-Jun N-terminal kinase kinase), one of the components of the stress response pathway (Ichijo, 1999; Tibbles and Woodgett, 1999). Such differences in response despite the use of a common export pathway underline the conclusion that the subcellular accumulation of any protein is the result of a complex interplay of factors affecting the nuclear import and export rates of individual proteins. In the case of Rev, the cellular context of the transport signals also appears to play a role in its subcellular localization. It was recently demonstrated in an astrocyte cell line that Rev accumulates in the cytoplasm but is not impeded from nuclear entry given the ability of LMB treatment to effect nuclear accumulation of the protein (Ludwig *et al.,* 1999). It remains to be determined whether the rate of nuclear import or nuclear export of Rev is affected in these cells. The basis for change in nuclear export rate of Rev described in this paper upon RNA polymerase inhibition, oxidative, and osmotic shock and upon inhibition of certain kinases remains to be determined since it cannot be correlated with alterations in phosphorylation of either Rev or CRM1 (Fig. 6). Since Rev is crucial to the growth of HIV, alteration of its location and activity is a potentially important mechanism by which new anti-viral strategies can be developed.

MATERIALS AND METHODS

Cell lines and transfections

The HeLaRev cell line used in some of the redistribution assays was generously provided by M. Malim and has been previously described (Meyer and Malim, 1994). This cell line was maintained in Isocove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 50 μg/ml gentamycin sulfate, 2.5 μg/ml amphotericin B, and 1 μg/ml amethopterin.

HeLa and Cos-7 cells were maintained in IMDM supplemented with 10% FBS, 50 μg/ml gentamycin sulfate,

and 2.5 μg/ml amphotericin B. For transient expression studies, vectors were introduced into either Cos-7 cells by the DEAE-dextran protocol, as previously described (Cullen, 1988), or HeLa cells by calcium phosphate transfection (Kriegler, 1990). Two days post-transfection, cells were used for analysis.

Expression constructs

The plasmids SVH6Rev and SVH6M10 have been previously described (Olsen *et al.,* 1990, 1991). The plasmid SVNLSH6Rev was generated by PCR using the 5′ primer, 5′-CCGAA TTCAT GCCTA AGAAG AAGAG GA AGG TT-GAA GACCC TGCAG GAAGA AGCGG AGC-3′, that contains the nuclear localization signal of the SV40 large T antigen and the 3′ primer, 5′-CCCCC CTCTA GATCT CTATT CTTTA GTTTC G-3′. For the PCR reaction, SVH6Rev was used as the template. The amplicon generated was digested with *Eco*RI and inserted into the *Eco*RI/*Sma*I sites of the vector SVpA, containing the SV40 early promoter, the small t intron and SV40 polyadenylation signal. The plasmid pDM128 has also been described (Hope *et al.,* 1990).

Drug treatments

Cells were treated with various drugs in the presence of 20 μg/ml cycloheximide (Sigma) for 30 min or 1 or 2 h as described. The drugs used in the assays include: actinomycin D (Sigma), DRB (Sigma), hydrogen peroxide, sorbitol, anisomycin (Sigma), sodium orthovanadate (Fisher Scientific), okadaic acid (Sigma), wortmannin (Sigma), the MEKI inhibitor PD98059 (New England Biolabs), herbimycin A (Sigma), genistein (Sigma), staurosporine (Sigma), H89 dihydrochloride (Calbiochem), SB 203580 (Calbiochem), and A3 hydrochloride (Calbiochem). Leptomycin B, a generous gift of Novartis and Minoru Yoshida (Nishi *et al.,* 1994; Kudo *et al.,* 1998), was used at 200 nM where indicated.

Immunofluorescence and immunoprecipitation

Cells grown on coverslips were processed for immunofluorescence as previously described (Perkins *et al.,* 1989). Polyclonal antibody to Rev was generously provided by C. A. Rosen. Monoclonal antibodies 4F4 and 4B10 to hnRNP C and hnRNP A1, respectively, were generously provided by G. Dreyfuss. Polyclonal antibody to CRM1 was generously provided by G. Grosveld. Fluorescein-labeled anti-rabbit and Texas-Red-labeled anti-mouse antibodies (Jackson ImmuoResearch, Bio/Can Scientific) were used to detect the polyclonal and monoclonal antibodies, respectively. Immunofluorescence was detected using a Leica DMR microscope at ×630 magnification.

Cells were labeled for immunoprecipitation studies with either ^{35}S-Translabel (ICN) or [^{32}P]orthophosphate (Amersham). Cells were washed twice with TBS and incubated in Dulbecco's modified Eagle medium (DMEM) without L-glutamine and L-methionine (GibcoBRL) supplemented with 10% FBS for 1 h prior to addition of the translabel for 24 h. Cells were washed twice with TBS and incubated in DMEM without sodium phosphate and sodium pyruvate (GibcoBRL) supplemented with 0.5% FBS for 1 h prior to addition of the orthophosphate for 1 h. During 1 h of labeling, cells were treated with the various drugs as indicated and subsequently harvested in RIPA buffer (150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5). Samples were processed for immunoprecipitation as previously described (Cochrane *et al.,* 1989), using polyclonal antibody to Rev or CRM1. Immunoprecipitated material was fractionated on 5% or 12.5% SDS–PAGE gels and detected by exposure to Phosphor Imager screens (Molecular Dynamics).

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